On the recalcitrant use of Arnon's method for chlorophyll determination

Raquel Esteban[1*], Jose I. García-Plazaola[1], Antonio Hernández[1], Beatriz Fernández-Marín[1]

[1]*Plant Biology and Ecology Department, c/Sarriena s/n. Apdo 644 48080.University of the Basque Country (UPV/EHU), Spain*

*corresponding author: raquel.esteban@ehu.es; telephone: +34946015012

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Abstract

- One of the most used protocols in Plant Biology for the simultaneous quantification of chlorophyll *a* and *b* is the one described by Arnon in 1949, which is based on old and inaccurate molar extinction coefficients calculated for acetone 80% as solvent.
- The quantification of chlorophylls by this method is incorrect. Thus, the use of it leads to important erroneous measurements and conclusions in many areas of plant biology.
- Surprisingly, and suddenly in the last decade, the use of Arnon´s method reached its highest historical rates.
- By this letter, we analyze the potential reasons for the recalcitrant use of this method and aim to send a clear message to the scientific community in order to replace the Arnon's equations by other more precise methods.

Keywords: Accuracy, Arnon, chlorophyll a, chlorophyll b, determinations.

Some of the most widely-utilized measurements in plant science research, such as total protein content or chlorophyll determination, are both straightforward and indispensable. The accuracy of the techniques employed for the quantification of these compounds is an important part of lab work, which will compromise the further determinations and even the conclusions of many of the articles published now and in the future. For the topic of this letter, chlorophyll determination, a complete set of methodologies, from remote sensing to chromatographic analyses, are available (Blackburn & Ferwerda, 2008; Cortazar *et al.*, 2015; Junker & Ensminger, 2016). Among this broad array of methodologies, one of the most (if not the most) successful protocol for the simultaneous quantification of chlorophyll *a* and *b* is the one described by Arnon in the middle of last century (Arnon, 1949). Evidence of its success is the fact that it has received almost 14000 citations up to now and has been in the top 100 most cited papers of all time (Garfield, 1990) for decades, a position only recently lost (it ranks 139th, July 2017). Even so, Arnon's paper is frequently cited even now (actually, it is one of the 4 most-cited papers ever in the area of Plant Science, according to the Web of Sciences, 2017). In fact, the highest number of citations was recorded in 2014, 2015 and 2016 (Fig. 1). Interestingly, chlorophyll determination was not the main achievement of this paper, which focused on copper metabolism in chloroplasts. However the equations proposed for chlorophyll measurement became the reason for its success. In fact, his previous Nature paper on copper (Arnon, 1948) has only received 15 citations since its publication. Despite their great success in numeric terms and in spite of the fact that they enjoy such a huge following, Arnon's equations are based on old and inaccurate molar extinction coefficients calculated for acetone 80% as solvent.

The imprecise of this method was evidenced decades later, when newer and more precise spectrometric determinations established the basis for several other methods subsequently published (Jeffrey & Humphrey, 1975; Lichtenthaler, 1987; Porra *et al.*, 1989; Wellburn, 1994). These methods described equations for a wide range of solvents and used atomic absorption spectrometry and pure standards to quantify the chlorophyll content exactly. The use of Arnon's method instead of more accurate methods is not trivial, and

it leads to substantial and important errors as was reported by Porra *et al.* (1989), particularly large for the calculation of the ratio Chl a/b. This parameter is of paramount importance as a robust indicator of significant processes such as the circadian rhythms of photosynthesis (García-Plazaola *et al.*, 2017), the evolution of pigment composition in protein-chlorophyll complexes (Kunugi *et al.*, 2016), the light environment in which a plant develops (Hogewoning *et al.*, 2012) or the acclimation to temperature stress (Fernández-Marín *et al.*, 2017). Additionally, the Chl a/b ratio is also a differentiating parameter among functional groups (Esteban *et al.*, 2015). Thus, worryingly, the use of Arnon's procedure could lead to flawed conclusions regarding many different plant functional and evolutionary aspects.

A deeper chronological analysis of the citations of Arnon's paper reveals an unusual bimodal pattern (Fig. 1). There was an initial and progressive rise that peaked in the 80s, when the inaccuracy of Arnon's equations was evidenced. This spike in the number of citations was followed by a sustained decrease, reaching a low point in 2009 with less than 100 citations. However, after this minimum, a second rise in Arnon's citations is now taking place. This pattern contrasts with that of almost all the papers within the top-100. Many of them describe basic biochemical techniques (protein quantification, PCR, RNA isolation…). Typically, the historical citation of these top-100 papers follows (i) either a mono-modal citation-pattern (i.e. they are intensively cited during a variable period of time following publication and later become obsolete, beginning to decline when a new alternative and more precise technology is made available), which is the case of 27% of current top-100 manuscripts, or (ii) a continuous rise (i.e. they are still active and their number of citations is still increasing) as is the case of 52% of top-100 manuscripts (Van Noorden *et al.*, 2014). In fact, among the afore-mentioned top-100, only the classical Bradford method for protein assay (Bradford, 1976) exhibits the same bimodal distribution of citations.

The question is, therefore, why has the use of a protocol considered inaccurate and imprecise, been resurrected during the last decade, being used even in studies using cutting-edge technologies? This was noticed 15 years ago (Porra, 2002), when this author considered as "contrary to reason that so many researchers continue to use Arnon's equations". Just at the onset of this sudden

rise in Arnon's citations, a further warning was made by Merchant (2010), who considered that the recalcitrant use of this method was "the result of a 'hand-me-down' rather than the obstinate refusal to accept improved procedures". Despite these clear messages, Arnon's protocol has been propelled to its highest rate of citations per year. It might be considered that the enhanced use of Arnon's method may simply be due to the global rise in the rate of publications during the last decade. In fact, the number of papers included in the "Plant Science" category has increased by 45% over the last 7 years and studies including the term "chlorophyll" has increased at approximately the same rate (Fig 1). However, the proportion of papers including the term "chlorophyll" that cited Arnon followed a much faster trend, increasing 5-fold (from 1,6 to 7,9) between 2009 and 2016. When analyzing the origin of the "second rise" in Arnon citations (period 2010-2017), we found a clear dividing line between the countries of origin of authors (Fig. 2). There was a much higher frequency of use in developing and low-income countries than in developed nations with increased resources for investment in I+D.

The rising number of new journals of debatable scientific quality could be argued as a possible reason for the second spike in Arnon's citations (Bohannon, 2013; Fernandez-Marín *et al.*, 2015). Nevertheless, a deeper analysis of the most recent citations to Arnon 1949 (i.e. a random selection of 100 papers published in the period 2014-2017) provides evidence against this assumption. In fact, 33% of the manuscripts were published in journals with IF>4, and 64% in journals of first quartile in their respective areas (Fig. 3). Worryingly, and in agreement with Fernandez-Marín *et al.* (2015), what is surprising is the inability of journals to filter out unreliable data.

It being clear that the increased use of Arnon's method is not by chance, but presumably intentional, the simple question that arises is why so many scientists have started to use this inaccurate protocol for their research. Whether this unfortunate case is an exception in modern Plant Biology or otherwise, it represents the visible tip of a much larger iceberg, and requires the attention of the plant biologists' community. Unfortunately, recent publications provide evidence that suggests that this problem is indeed "tip of the iceberg". An alarming level of inaccuracy in the use of methods in Environmental Sciences in general (Tran *et al.*, 2017) and regarding plant pigment

quantification in particular (Fernandez-Marín *et al.*, 2015) has been denounced recently. In fact, even in the manuscripts that follow Arnon's procedure, astonishingly, a significantly high proportion (11%) included additional mistakes, such as the use of a different extraction method (instead of the 80% acetone used by Arnon), or the measuring of absorbance at different wavelengths (than the 663 and 645 indicated by Arnon) or incorrect coefficients. This leads to a bizarre type of error that might be termed "nested error" (an error inside an error).

Overall, bearing in mind that the publication of correct and reliable data should be a prerequisite and the responsibility of authors, readers, reviewers and editors, more rigorous measures of quality seem to be necessary. From the present communication, at least, a clear message needs to be sent: "replace Arnon's equations with other, more precise and accurate equations".

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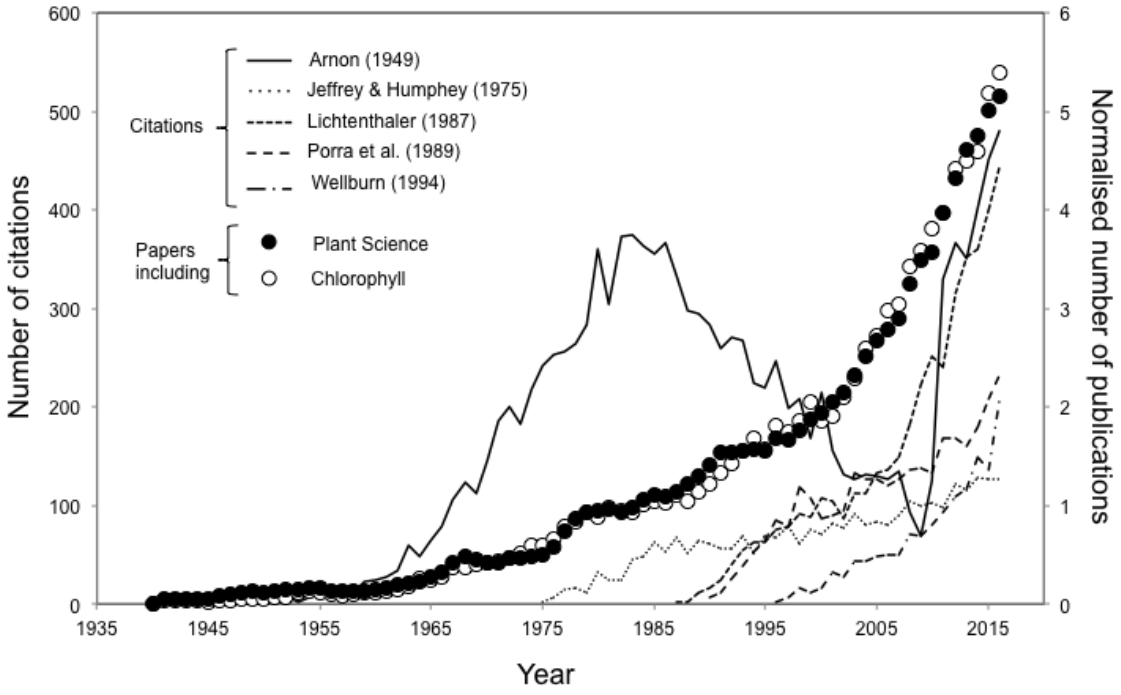

Figure 1. Citations per year of the most widely employed protocols for chlorophyll determination (left scale), normalised number of publications (publications by year divided by the total number of papers published between 1940 and 2016) within the area of Plant Science (filled dots) and normalised number of publications including the term “chlorophyll” (empty dots) (right scale).

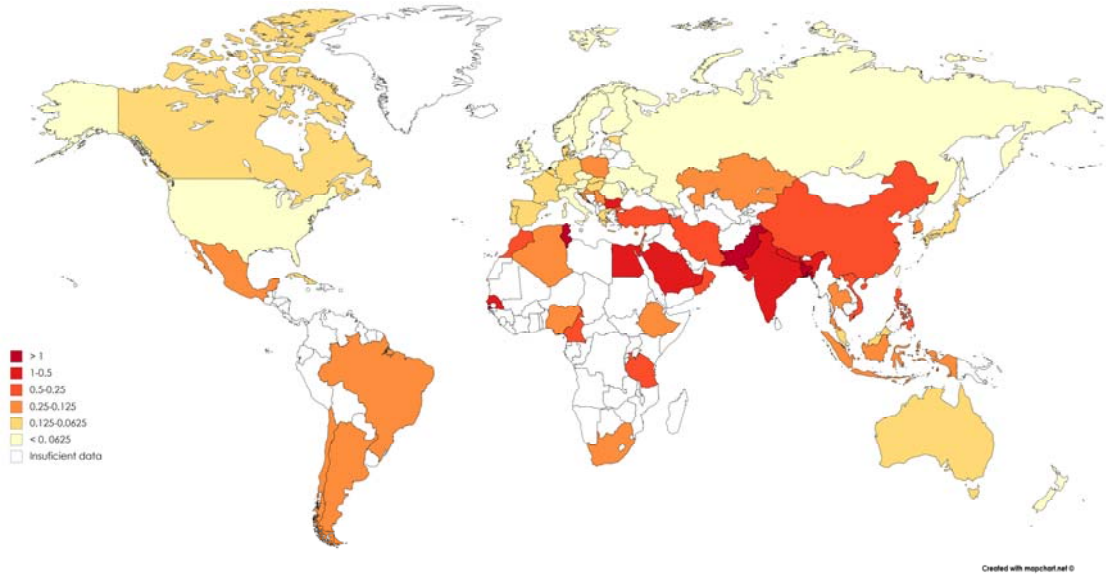

Figure 2. Normalised citation number of the publications, in which Arnon´s method is used for chlorophyll determination, during the period 2010-2017 along 70 countries. Normalised citation for each country is calculated as the number of publications citing Arnon divided by the total number of papers published in the research field of knowledge: SCIENCE TECHNOLOGY (x1000). Source: Web of Science.

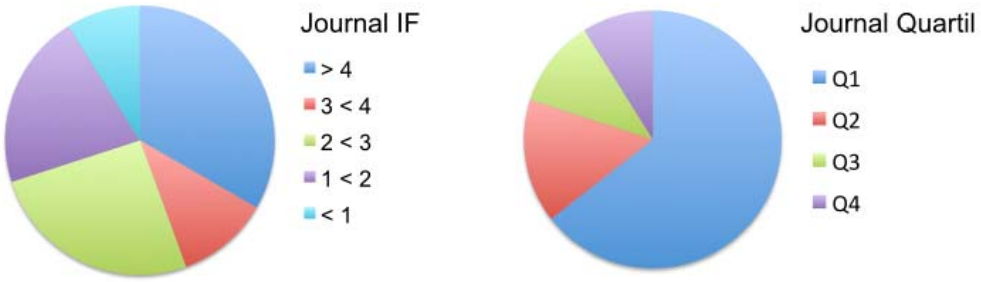

Figure 3. Analysis of the quality of the journals in which Arnon 1949 was recently cited. 100 manuscripts were included in the analysis covering the 20 most recent works of the last 5 years (source: WoS, June 2017). On the left, journals are grouped according to their IF. On the right, journals are classified according to the quartile in their respective areas. Highest quartile was used for journals appearing in several areas.